FORM PTO-1390 (REV. 5-93) U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE	ATTORNEY'S DOCKET NUMBER
TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371	ST94065-US U.S. APPLICATION NO. (If known, See 37 C.F.R. 1.5) 08/776786

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/FR95/01002	26 July 1995	12 August 1994

TITLE OF INVENTION

ADENOVIRUS COMPRISING A GENE CODING FOR GLUTATHIONE PEROXIDASE

APPLICANT(S) FOR DO/EO/US

Martine BARKATS, Jacques MALLET and Frédéric REVAH

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. [X] This is a FIRST submission of items concerning a filing under 35 U.S.C. 371.
2. [ ] This is a SECOND or SUBSEQUENT submission of items concerning a filing under 35 U.S.C. 371.
3. [X] This is an express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).
4. [X] A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
5. [X] A copy of the International Application as filed (35 U.S.C. 371(c)(2))
   a. [ ] is transmitted herewith (required only if not transmitted by the International Bureau).
   b. [X] has been transmitted by the International Bureau.
   c. [ ] is not required, as the application was filed in the United States Receiving Office (RO/US)
6. [X] A translation of the International Application into English (35 U.S.C. 371(c)(2)).
7. [X] Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))
   a. [ ] are transmitted herewith (required only if not transmitted by the International Bureau).
   b. [ ] have been transmitted by the International Bureau.
   c. [ ] have not been made; however, the time limit for making such amendments has NOT expired.
   d. [X] have not been made and will not be made.
8. [ ] A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. [X] An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)), and Power of Attorney, unsigned.
10. [ ] A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11. to 16. below concern other document(s) or information included:

11. [ ] An information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. [ ] An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. [X] A FIRST preliminary amendment.
    [ ] A SECOND or SUBSEQUENT preliminary amendment.
14. [ ] A substitute specification.
15. [ ] A change of power of attorney and/or address letter.
16. [ ] Other items or information:

CERTIFICATION UNDER 37 CFR 1.10

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I hereby certify that this paper (along with any referred to as being attached or enclosed) is being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 CFR 1.10 on the date indicated above and is addressed to Box PCT, Assistant Commissioner for Patents, Washington, D.C. 20231, Attn. EO/US

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U.S. APPLICATION NO. (If Known, see C.F.R. 1.5)	INTERNATIONAL APPLICATION NO. PCT/FR95/01002	ATTORNEY'S DOCKET NUMBER ST94065-US

17. [X] The following fees are submitted:

	CALCULATIONS	PTO use only
Basic National Fee (37 CFR 1.492(a)(1)-(5): Search Report has been prepared by the EPO or JPO.....$ 910.00		
International preliminary examination fee paid to USPTO (37 CFR 1.482) .....$ 700.00		
No international preliminary examination fee paid to USPTO (37 CFR 1.482) but international search fee paid to USPTO (37 CFR 1.445(a)(2))....$ 770.00		
Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO.....$1040.00		
International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(2)-(4).....$ 96.00		
ENTER APPROPRIATE BASIC FEE AMOUNT =	$ 910.00	
Surcharge of $130.00 for furnishing the oath or declaration later than [ ] 20 [ ] 30 months from the earliest claimed priority date (37 CFR 1.492(e)).	$	

Claims	Number Filed	Number Extra	Rate		
Total Claims	29 -20 =	9	X $ 22.00	$ 198.00	
Independent Claims	2 - 3 =	0	X $ 80.00	$	
Multiple dependent claim(s) (if applicable)			+ $260.00	$	
TOTAL OF ABOVE CALCULATIONS =				$ 1108.00	
Reduction by 1/2 for filing by small entity, if applicable. Verified Small Entity statement must also be filed. (Note 37 CFR 1.9, 1.27, 1.28).				$	
SUBTOTAL =				$ 1108.00	
Processing fee of $130.00 for furnishing the English translation later the [ ] 20 [ ] 30 months from the earliest claimed priority date (37 CFR 1.492(f)).			+	$	
TOTAL NATIONAL FEE =				$ 1108.00	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). $40.00 per property +				$	
TOTAL FEES ENCLOSED =				$ 1108.00	
				Amount to be: refunded	$
				charged	$

a. [ ] A check in the amount of $_____ to cover the above fee is enclosed.

b. [X] Please charge my Deposit Account No. 18-1982 in the amount of $ 1108.00 to cover the above fees. A duplicate copy of this sheet is enclosed.

c. [X] The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 18-1982. A duplicate copy of this sheet is enclosed.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:
Martin F. Savitzky, Esquire
Rhone-Poulenc Rorer Inc.
Legal-Patents, #3C43
P.O. Box 5093
Collegeville, PA 19426-0997
Telephone: (610) 454-3816
Facsimile: (610) 454-3808

Signature

Martin F. Savitzky
Name

29,699
Registration Number

Date February 11, 1997

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re the Application of:	BARKATS et al.	Group Art Unit:
Serial No.:	To Be Assigned	Examiner:
	U.S. National Stage of PCT/FR95/01002	
Filed:	Concurrently Herewith	

For: Adenovirus Comprising A Gene Coding For Glutathione Peroxidase

To: The Honorable Commissioner of Patents and Trademarks
Washington, D.C. 20231

CERTIFICATE OF MAILING (37 CFR § 1.10)

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Paula L. Dickey — (Type or print name of person mailing paper)

Paula L. Dickey — (Signature of person mailing paper)

PRELIMINARY AMENDMENT

Please enter the following amendment to the English Translation of the International Application before examining this application.

In the Claims

Please cancel claims 1-26 without prejudice.

Please add the following new claims 27-55:

27. A replication defective recombinant adenovirus comprising at least one DNA sequence encoding all or an active part of a glutathione peroxidase, or a derivative thereof.

28. An adenovirus according to claim 27, wherein the DNA sequence is a cDNA sequence.

29. An adenovirus according to claim 27, wherein the DNA sequence is a gDNA sequence.

30. An adenovirus according to claim 27, wherein the DNA sequence encodes a bovine glutathione peroxidase.

31. An adenovirus according to claim 27, wherein the DNA sequence encodes a human glutathione peroxidase.

32. A replication defective recombinant adenovirus comprising at least one DNA sequence encoding an antisense sequence capable of controlling the expression of a gene encoding glutathione peroxidase.

33. An adenovirus according to claim 32, wherein the antisense sequence is an antisense RNA capable of controlling the translation of the mRNA for a glutathione peroxidase.

34. An adenovirus according to claim 27, wherein the DNA sequence is under the control of signals controlling expression in target cells.

35. An adenovirus according to claim 34, wherein the signal is a viral promoter.

36. An adenovirus according to claim 35, wherein the promoter is selected from the group consisting of E1A, MLP, CMV and RSV-LTR promoters.

37. An adenovirus according to claim 27, comprising a gDNA or cDNA sequence encoding a bovine glutathione peroxidase under the control of an RSV-LTR promoter.

38. An adenovirus according to claim 27, comprising a gDNA or cDNA sequence encoding a human glutathione peroxidase under the control of an RSV-LTR promoter.

39. An adenovirus according to claim 27, lacking regions of the genome necessary for its replication in a target cell.

40. An adenovirus according to claim 39, comprising ITRs and a sequence permitting encapsidation, wherein the E1 gene and at least one of the E2, E4, or L1-L5 genes are not functional.

41. An adenovirus according to claim 39, wherein said adenovirus is an Ad 2 or Ad 5 human adenovirus or a CAV-2 canine adenovirus.

42. A method for the treatment and/or prevention of a neurodegenerative disease comprising administering to a patient an adenovirus according to claim 27.

43. A method according to claim 42, wherein the neurodegenerative disease is selected from the group consisting of Parkinson's disease, Alzheimer's disease, Huntington's disease, ALS, trisomy 21, atherosclerosis, cardiovascular diseases, cirrhosis of the liver, diabetes, the formation of cataracts, cerebral ischaemia, cranial traumas, respiratory distress syndrome (ARDS), cancers and the aging process.

44. A pharmaceutical composition comprising one or more replication defective recombinant adenoviruses according to claim 27.

45. A pharmaceutical composition according to claim 44, in injectable form.

46. A pharmaceutical composition according to claim 44, comprising between 10^4 and 10^{14} pfu/ml of defective recombinant adenoviruses.

47. A pharmaceutical composition according to claim 46, comprising between 10^6 to 10^{10} pfu/ml of defective recombinant adenoviruses.

48. A mammalian cell infected with one or more defective recombinant adenoviruses according to claim 27.

49. A mammalian cell according to claim 48, wherein said cell is a human cell.

50. A mammalian cell according to claim 49, wherein said cell is a retinal cell, fibroblast, myoblast, hepatocyte, endothelial cell, glial cell or keratinocyte.

51. An implant comprising a cell according to claim 48 and an extracellular matrix.

52. An implant according to claim 51, wherein the extracellular matrix comprises a gelling compound.

53. An implant according to claim 52, wherein the gelling compound is selected from the group consisting of collagen, gelatin, glucosaminoglycans, fibronectin, agarose and lectins.

54. An implant according to claim 51, wherein the extracellular matrix comprises a support for anchorage of infected cells.

55. An implant according to claim 54, wherein the support comprises polytetrafluoroethylene fibres.

REMARKS

Claims 1-26 have been cancelled and rewritten as new claims 27-55, in order to conform with US patent practice. Support for the new claims is found in the claims as originally filed. No new matter has been added.

Respectfully submitted,

Rhône-Poulenc Rorer Inc.
Legal-Patents #3C43
P.O. Box 5093
Collegeville, PA 19426-0997
Telephone: (610) 454-3816
Facsimile: (610) 454-3808

Dated: Feb. 11, 1997

Martin Savitzky

Martin F. Savitzky
Attorney for Applicants
Registration No. 29,699

ADENOVIRUS COMPRISING A GENE CODING FOR GLUTATHIONE PEROXIDASE

The present invention relates to recombinant adenoviruses comprising a DNA sequence encoding glutathione peroxidase and its uses in gene therapy.

Glutathione peroxidase is one of the enzymes which are actively involved in the regulation of the concentration of oxygen-derived free radicals formed during various physiological or pathological processes.

Normally, the formation of these radicals, which are highly reactive, such as the superoxide anion, hydrogen peroxide and the hydroxyl radical is controlled as follows: superoxide anion is rapidly converted to hydrogen peroxide, by means of superoxide dismutase, then this hydrogen peroxide is converted to oxygen and water, by catalase or in particular glutathione peroxidase.

Usually, these enzymes are present in practically all tissues.

However, under certain conditions, these regulatory mechanisms are not totally efficient. In particular, there may be a disequilibrium between their respective concentrations, for example an excessive superoxide dismutase concentration compared with the available quantity of glutathione peroxidase, leading to a pathological production of hydrogen peroxide and of free radicals (hydroxyl radicals in particular).

These free radicals may directly induce a

peroxidation of membrane lipids, inactivate enzymes by peroxidizing their sulphydryl groups, depolymerize polysaccharides and/or damage nucleic acids, causing in all cases serious pathologies. They may thus be responsible for inflammations, emphysemas, neoplasms and/or retinopathies. They also appear to be involved in atherosclerosis, cerebral ischaemia, cranial traumas, respiratory distress syndrome, cardiovascular diseases, diabetes, cirrhosis of the liver and formation of cataracts as well as in the aging process. Free radicals are also thought to be linked to the apoptosis process and could be involved in the cell death accompanying the acquired immunodeficiency syndrome (AIDS), [The J. of Biol. Chem., 269, 2(14), 798-801, (1994)]. More recently, it has been demonstrated that reactions between these radicals or with neurotransmitters led to the formation of endogenous neurotoxins. Free radicals are therefore also involved in neurological pathologies such as Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis (ALS) and/or trisomy 21.

Consequently, it would be particularly valuable to have available nowadays medicinal products which can increase or regulate the glutathione peroxidase concentration in the body and which are therefore effective for treating all the abovementioned pathologies.

The present invention is precisely consists

in the development of vectors which are particularly efficient for delivering in vivo and in a localized manner, therapeutically active quantities of the specific gene encoding glutathione peroxidase or one of its derivatives.

In the corresponding application no. PCT/EP93/02519, it has been shown that adenoviruses could be used as vector for the transfer of a foreign gene in vivo into the nervous system and the expression of the corresponding protein.

The present invention relates more particularly to new constructs which are particularly suitable and efficient for controlling the expression of glutathione peroxidase.

More precisely, it relates to a recombinant adenovirus comprising a DNA sequence for controlling the expression of a glutathione peroxidase, its use for therapeutic treatments and/or the prevention of various pathologies.

The Applicant has thus demonstrated that it is possible to construct recombinant adenoviruses containing a sequence encoding a glutathione peroxidase, to administer these recombinant adenoviruses in vivo, and that this administration allows a stable and localized expression of therapeutically active quantities of glutathione peroxidase in vivo.

A first subject of the invention therefore

consists in a defective recombinant adenovirus comprising at least one DNA sequence encoding all or an active part of a glutathione peroxidase or one of its derivatives.

For the purposes of the present invention, glutathione peroxidase designates any enzyme having glutathione peroxidase activity. By way of illustration of these enzymes, there may in particular be mentioned in man the glutathione peroxidases GPX1, GPX2, GPX3 and GPX4. GPX1 and GPX4 are expressed in most tissues with a clear predominance in the erythrocytes, the liver and the kidneys for GPX1 (Chambers et al; EMBO J 5: 1221-1227 (1986)) and in the testicles for GPX4 [Roveri et al; J. Biol. Chem. 267:6142-6146 (1992)]. GPX3 is produced in the kidneys, the lungs, the heart, the breast, the placenta as well as in the liver (Chu et al. Blood 79: 3233_3238 (1992)) as for GPX2, it has mainly been demonstrated in the gastrointestinal tissues and in the liver [Chu et al. J. Biol. Chem. 268: 2571-257 (1993)].

The glutathione peroxidase produced within the framework of the present invention may be a human or animal glutathione peroxidase. It may in particular be bovine glutathione peroxidase.

The DNA sequence encoding glutathione peroxidase, which is used within the framework of the present invention may be a cDNA, a genomic DNA (gDNA), or a hybrid construct consisting for example of a cDNA

into which one or more introns would be inserted. The nucleic sequence of the cDNA encoding human glutathione peroxidase has been described by [Mullenbach et al., Oxy-Radicals in Molecular Biology and Pathology, 313-326, (1988)]. It may also be synthetic or semisynthetic sequences.

In a particularly advantageous manner, a cDNA or a gDNA is used.

According to a preferred embodiment of the invention, it is a genomic DNA (gDNA) sequence encoding a glutathione peroxidase. Its use may allow an enhanced expression in human cells.

Of course, prior to its incorporation into an adenovirus vector according to the invention, the DNA sequence may be advantageously modified, for example by site-directed mutagenesis, in particular for the insertion of appropriate restriction sites. The sequences described in the prior art are indeed not constructed for a use according to the invention, and prior adaptations may prove necessary in order to obtain high expression levels.

For the purposes of the present invention, derivative is understood to mean any sequence obtained by modification and encoding a product which preserves at least one of the biological properties of glutathione peroxidase. Modification should be understood to mean any mutation, substitution, deletion, addition or modification of a genetic and/or

chemical nature. These modifications can be performed by techniques known to persons skilled in the art (see general molecular biology techniques below). The derivatives according to the invention can also be obtained by hybridization from nucleic acid libraries, using as probe the glutathione peroxidase native sequence or a fragment thereof.

These derivatives are especially molecules having a higher affinity for their binding sites, sequences allowing an enhanced expression in vivo, molecules having a greater resistance to proteases, molecules having a higher therapeutic efficacy or fewer side effects, or possibly new biological properties.

Among the preferred derivatives, there may be mentioned more particularly natural variants, molecules in which one or more residues have been substituted, derivatives obtained by deletion of regions having little or no involvement in the interaction with the binding sites considered or expressing an undesirable activity, and derivatives containing additional residues compared with the native sequence, such as for example a secretory signal and/or a joining peptide.

The DNA sequence, encoding all or part of a glutathione peroxidase or one of its derivatives, may also be an antisense sequence whose expression in the target cell makes it possible to control the expression of this enzyme. Preferably, the heterologous DNA sequence contains a gene encoding an antisense RNA

capable of controlling the translation of the corresponding mRNA. The antisense sequence may be all or only part of the DNA sequence encoding a glutathione peroxidase, inserted in the reverse orientation in the vector according to the invention.

According to one embodiment of the invention, the DNA sequence encoding a glutathione peroxidase or one of its derivatives can also integrate a secretory signal which makes it possible to direct the synthesized glutathione peroxidase in the secretory pathways of the infected cells. In this manner, the synthesized glutathione peroxidase is advantageously released into the extracellular compartments.

Advantageously, the glutathione peroxidase encoding sequence is placed under the control of signals allowing its expression in the target cells. Preferably, these are heterologous expression signals, that is to say signals which are different from those naturally responsible for the expression of glutathione peroxidase. They may be in particular sequences responsible for the expression of other proteins, or of synthetic sequences. In particular, they may be promoter sequences of eukaryotic or viral genes. For example, they may be promoter sequences derived from the genome of the cell which it is desired to infect. Likewise, they may be promoter sequences derived from the genome of a virus, including the adenovirus used. In that respect, there may be mentioned for example the

E1A, MLP, CMV, RSV-LTR promoters and the like. In addition, these expression sequences can be modified by addition of activation or regulatory sequences or of sequences allowing a tissue-specific expression. It may be particularly advantageous to use expression signals which are specifically or predominantly active in the target cells, so that the DNA sequence is expressed or produces its effect only when the virus has indeed infected a target cell.

In a first specific embodiment, the invention relates to a defective recombinant adenovirus comprising a cDNA or DNA8 sequence encoding a bovine glutathione peroxidase under the control of the RSV-LTR promoter.

In another specific embodiment, the invention relates to a defective recombinant adenovirus comprising a gDNA sequence encoding human glutathione peroxidase under the control of the RSV-LTR promoter.

A particularly preferred embodiment of the present invention consists in a defective recombinant adenovirus comprising the ITR sequences, a sequence allowing encapsidation, a DNA sequence encoding human glutathione peroxidase or a derivative thereof under the control of a promoter allowing predominant expression in the target tissues and in which the E1 gene and at least one of the E2, E4, L1-L5 genes is not functional.

The defective adenoviruses according to the

invention are adenoviruses which are incapable of autonomously replicating in the target cell. Generally, the genome of the defective adenoviruses used within the framework of the present invention therefore lacks at least the sequences necessary for the replication of the said virus in the infected cell. These regions can be either removed (completely or partially), or rendered nonfunctional, or substituted with other sequences and especially with the glutathione peroxidase encoding DNA sequence.

Preferably, the defective virus of the invention conserves the sequences of its genome which are necessary for the encapsidation of the viral particles. Still more preferably, as indicated above, the genome of the defective recombinant virus according to the invention comprises ITR sequences, a sequence allowing encapsidation, the nonfunctional E1 gene and at least one of the nonfunctional E2, E4, L1-L5 genes.

There are various serotypes of adenoviruses, whose structure and properties vary somewhat. Among these serotypes, the use of type 2 or 5 human adenoviruses (Ad 2 or Ad 5) or of adenoviruses of animal origin (see Application FR 93 05954) is preferred within the framework of the present invention. Among the adenoviruses of animal origin which can be used within the framework of the present invention, there may be mentioned adenoviruses of canine, bovine, murine [example: MAV1, Beard et al.,

Virology 75 (1990) 81], ovine, porcine, avian or even simian (example: SAV) origin. Preferably, the adenovirus of animal origin is a canine adenovirus, more particularly a CAV2 adenovirus [Manhattan strain or A26/61 (ATCC VR-800) for example]. Preferably, adenoviruses of human or canine or mixed origin are used within the framework of the invention.

The defective recombinant adenoviruses according to the invention can be prepared by any technique known to persons skilled in the art (Levrero et al., Gene 101 (1991) 195, EP 185 573; Graham, EMBO J. 3 (1984) 2917). In particular, they can be prepared by homologous recombination between an adenovirus and a plasmid carrying, inter alia, the glutathione peroxidase encoding DNA sequence. The homologous recombination occurs after co-transfection of the said adenoviruses and plasmid into an appropriate cell line. The cell line used should preferably (i) be transformable by the said elements and (ii) contain the sequences capable of complementing the defective adenovirus genome part, preferably in integrated form in order to avoid risks of recombination. As an example of a cell line, there may be mentioned the human embryonic kidney line 293 (Graham et al., J. Gen. Virol. 36 (1977) 59) which contains especially, integrated into its genome, the left-hand part of the genome of an Ad5 adenovirus (12%). Strategies for constructing vectors derived from adenoviruses have

also been described in Applications Nos. FR 93 05954 and FR 93 08596 which are incorporated into the present application by reference.

Next, the adenoviruses which have multiplied are recovered and purified according to conventional molecular biology techniques as illustrated in the examples.

The particularly advantageous properties of the vectors of the invention stem especially from the construct used (defective adenovirus, deleted of certain viral regions), the promoter used for the expression of the glutathione peroxidase encoding sequence (viral or tissue-specific promoters preferably), and methods for administering the said vector, allowing efficient expression of the said enzyme in the appropriate tissues.

The present invention also relates to any use of an adenovirus as described above for the preparation of a pharmaceutical composition intended for the treatment and/or prevention of the abovementioned pathologies. More particularly it relates to any use of these adenoviruses for the preparation of a pharmaceutical composition intended for the treatment and/or prevention of neurodegenerative diseases such as for example Parkinson's disease, Alzheimer's disease, amyotrophic lateral sclerosis (ALS) and trisomy 21. They can also be advantageously used in the treatment of atherosclerosis, cardiovascular diseases, cirrhosis

of the liver, diabetes, formation of cataracts, cerebral ischaemia, cranial traumas, respiratory distress syndrome (ARDS), diseases linked to an immune deficiency, cancers as well as in the aging process.

The present invention also relates to a pharmaceutical composition comprising one or more defective recombinant adenoviruses as described above. These pharmaceutical compositions may be formulated for topical, oral, parenteral, intranasal, intravenous, intramuscular, subcutaneous, intraocular or transdermal administration and the like. Preferably, the pharmaceutical compositions of the invention contain a pharmaceutically acceptable vehicle for an injectable formulation, especially for a direct injection into the patient. These may be in particular isotonic sterile solutions, or dry, especially freeze-dried, compositions which, upon addition, depending on the case, of sterilized water or physiological saline, allow the preparation of injectable solutions.

In this respect, the invention also relates to a method for treating neurodegenerative diseases comprising the administration, to a patient, of a recombinant adenovirus as defined above. More particularly, the invention relates to a method for treating neurodegenerative diseases comprising the stereotaxic administration of a recombinant adenovirus as defined above.

The doses of defective recombinant adenovirus

used for the injection can be adjusted according to various parameters, and especially according to the mode of administration used, the relevant pathology or even the desired duration of treatment. Generally, the recombinant adenoviruses according to the invention are formulated and administered in the form of doses of between 10^4 and 10^{14} pfu/ml, and preferably 10^6 to 10^{10} pfu/ml. The term pfu (plaque forming unit) corresponds to the infectivity of a virus solution, and is determined by infecting an appropriate cell culture and measuring, generally after 48 hours, the number of plaques of infected cells. The techniques for determining the pfu titre of a viral solution are well documented in the literature.

Another subject of the invention relates to any mammalian cell infected by one or more defective recombinant adenoviruses as described above. More particularly the invention relates to any human cell population infected by these adenoviruses. This may be in particular fibroblasts, myoblasts, hepatocytes, keratinocytes, endothelial cells, glial cells and the like.

The cells according to the invention can be obtained from primary cultures. These can be collected by any technique known to persons skilled in the art and then cultured under conditions permitting their proliferation. As regards more particularly fibroblasts, these can be easily obtained from

biopsies, for example according to the technique described by Ham [Methods Cell. Biol. 21a (1980) 255]. These cells can be used directly for infection by adenoviruses, or preserved, for example by freezing, for establishing autologous libraries, for subsequent use. The cells according to the invention can also be secondary cultures which are obtained for example from pre-established libraries.

The cultured cells are then infected with the recombinant adenoviruses, so as to confer on them the capacity to produce glutathione peroxidase. The infection is carried out in vitro according to techniques known to persons skilled in the art. In particular, depending on the type of cells used and the desired copy number of virus per cell, persons skilled in the art can adjust the multiplicity of infection. It is clearly understood that these steps should be carried out under appropriate sterile conditions when the cells are intended for administration in vivo. The recombinant adenovirus doses used for the infection of the cells can be adjusted by persons skilled in the art according to the desired aim. The conditions described above for administration in vivo can be applied to infection in vitro.

Another subject of the invention relates to an implant comprising mammalian cells infected with one or more defective recombinant adenoviruses as described above, and an extracellular matrix. Preferably, the

implants according to the invention comprise 10^5 to 10^{10} cells. More preferably, they comprise 10^6 to 10^8 cells.

More particularly, in the implants of the invention, the extracellular matrix comprises a gelling compound and optionally a support permitting anchorage of the cells.

For the preparation of the implants according to the invention, various types of gelling agents can be used. The gelling agents are used for the inclusion of the cells in a matrix having the constitution of a gel, and to enhance the anchorage of the cells on the support, where appropriate. Various cell adhesion agents can therefore be used as gelling agents, such as especially collagen, gelatin, glucosaminoglycans, fibronectin, lectins, agarose and the like.

As indicated above the compositions according to the invention advantageously comprise a support permitting anchorage of the cells. The term anchorage designates any form of biological and/or chemical and/or physical interaction resulting in the adhesion and/or binding of the cells onto the support. Moreover, the cells can either cover the support used, or penetrate inside this support, or both. The use of a solid, non-toxic and/or biocompatible support is preferred within the framework of the invention. In particular, it is possible to use polytetrafluoroethylene (PTFE) fibres or a support of biological origin.

The implants according to the invention can be implanted at different sites in the body. In particular, the implantation can be carried out in the peritoneal cavity, in the subcutaneous tissue (suprapubic region, iliac or inguinal fossae, and the like), in an organ, a muscle, a tumour, the central nervous system or alternatively under a mucous membrane. The implants according to the invention are particularly advantageous in the sense that they make it possible to control the release of the therapeutic product in the body: this release is first determined by the multiplicity of infection and by the number of implanted cells. Next, the release can be controlled either by the removal of the implant, which permanently stops the treatment, or by the use of regulable expression systems, which make it possible to induce or to repress the expression of the therapeutic genes.

The present invention thus provides viral vectors which can be directly used in gene therapy, and which are particularly suitable and efficient for directing the expression of glutathione peroxidase in vivo. The present invention thus offers a new approach which is particularly advantageous for the treatment and/or prevention of many pathologies such as those mentioned above.

The adenoviral vectors according to the invention have, in addition, major advantages, linked especially to their very high efficiency of infection

of the target cells, which make it possible to achieve infections with small volumes of viral suspension. Furthermore, infection with the adenoviruses of the invention is highly localized at the site of injection, which avoids the risks of diffusion to the neighbouring cerebral structures. This treatment may apply both to man and to any animal such as ovines, bovines, murines, domestic animals (dogs, cats and the like), horses, fish and the like.

It is perfectly possible, in addition, to envisage a simultaneous administration of an adenovirus according to the invention with at least a second adenovirus containing a gene encoding one of the forms of superoxide dismutase or catalase.

The examples and the single figure are presented below as a guide and do not limit the scope of the invention.

FIGURE

Figure 1: representation of the enzymatic activity of the glutathione peroxidase obtained from cells 293 infected with 0 to 500 pfu/recombinant adenovirus cell encoding GPx (AdGPx) or β-galactosidase (Adβgal).

General molecular biology techniques

The methods conventionally used in molecular biology, such as preparative extractions of plasmid DNA, centrifugation of plasmid DNA in caesium chloride gradient, agarose or acrylamide gel electrophoresis,

purification of DNA fragments by electroelution, phenol or phenol-chloroform extraction of proteins, ethanol or isopropanol precipitation of DNA in saline medium, transformation in Escherichia coli and the like, are well known to persons skilled in the art and are widely described in the literature [Maniatis T. et al., "Molecular Cloning, a Laboratory Manual", Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., 1982; Ausubel F.M. et al. (eds), "Current Protocols in Molecular Biology", John Wiley & Sons, New York, 1987].

The pBR322- and pUC-type plasmids and the phages of the M13 series are of commercial origin (Bethesda Research Laboratories).

For the ligations, the DNA fragments can be separated according to their size by agarose or acrylamide gel electrophoresis, extracted with phenol or with a phenol/chloroform mixture, precipitated with ethanol and then incubated in the presence of phage T4 DNA ligase (Biolabs) according to the recommendations of the supplier.

The filling of the protruding 5′ ends can be performed with the Klenow fragment of E. coli DNA polymerase I (Biolabs) according to the specifications of the supplier. The destruction of the protruding 3′ ends is performed in the presence of phage T4 DNA polymerase (Biolabs) used according to the recommendations of the manufacturer. The destruction of the protruding 5′ ends is performed by a controlled

treatment with S1 nuclease.

Site-directed mutagenesis in vitro by synthetic oligodeoxynucleotides can be performed according to the method developed by Taylor et al. [Nucleic Acids Res. 13 (1985) 8749-8764] using the kit distributed by Amersham.

The enzymatic amplification of DNA fragments by the so-called PCR technique [Polymerase-catalyzed Chain Reaction, Saiki R.K. et al., Science 230 (1985) 1350-1354; Mullis K.B. and Faloona F.A., Meth. Enzym. 155 (1987) 335-350] can be performed using a DNA thermal cycler (Perkin Elmer Cetus) according to the specifications of the manufacturer.

The verification of the nucleotide sequences can be performed by the method developed by Sanger et al. [Proc. Natl. Acad. Sci. USA, 74 (1977) 5463-5467] using the kit distributed by Amersham.

Examples

Example 1: Procedure for the construction of the vector pLTRIX-bGPx

This vector contains the sequence encoding bovine GPx under the control of the RSV virus LTR, as well as sequences from the adenovirus which allow recombination in vivo. The cDNA used is described in [Mullenbach et al., Oxy-Radicals in Molecular Biology and Pathology, 313-326, (1988)].

The DNA is inserted into the BamHI site of a plasmid Bluescript. A polyadenylzation sequence was introduced

into the XhoI site of this plasmid. The latter is identified by SK-bGPx-PolyA.

The vector pLTRIX-bGPx is obtained by introducing an insert obtained by cleavage of SK-bGPx-PolyA into the EcoRV site of the plasmid pLTRIX.

Example 2: Construction of recombinant adenoviruses containing a sequence encoding bovine glutathione peroxidase.

The vector pLTRIX-bGPx is linearized and cotransfected with a deficient adenoviral vector, into the helper cells (line 293) providing in trans the functions encoded by the adenovirus E1 regions (E1A and E1B).

More precisely, the Ad-bGPx adenovirus was obtained by homologous recombination in vivo between the mutant adenovirus Ad-dl1324 (Thimmappaya et al., Cell 31 (1982) 543) and the vector pLTR IX-bGPx, according to the following procedure: the plasmid pLTR IX-bGPx and the Ad-dl1324 adenovirus, linearized by the enzyme ClaI, were cotransfected into the line 293 in the presence of calcium phosphate, so as to allow the homologous recombination. The recombinant adenoviruses thus generated were selected by plaque purification. After isolation, the recombinant adenovirus DNA was amplified in the cell line 293, thereby giving a culture supernatant containing the unpurified recombinant defective adenovirus having a titre of about 10^{10} pfu/ml.

The viral particles are then purified by gradient centrifugation.

Example 3: Control of the expression in vitro of GPx.

For each test, an extract (0.5% triton) is produced from 300,000 cells 293 infected with 0 to 500 pfu/recombinant adenovirus cell encoding GPx or β-galactosidase. The enzymatic activity of glutathione peroxidase is evaluated according to the procedure of Flohé and Günzler (1984, Methods in Enzymology, Vol; 105, pp 114-121). The oxidized glutathione (GSSG) formed during the GPx reaction is constantly reduced by an excess of glutathione reductase activity for a constant level of reduced glutathione (GSH). The simultaneous oxidation of NADPH is monitored by spectrophotometry.

Figure 1 presents the results obtained.

CLAIMS

1. Defective recombinant adenovirus comprising at least one DNA sequence encoding all or an active part of a glutathione peroxidase or one of its derivatives.

2. Adenovirus according to claim 1, characterized in that the DNA sequence is a cDNA sequence.

3. Adenovirus according to claim 1, characterized in that the DNA sequence is a gDNA sequence.

4. Adenovirus according to claim 1, 2 or 3, characterized in that the DNA sequence encodes a bovine glutathione peroxidase.

5. Adenovirus according to claim 1, 2 or 3, characterized in that the DNA sequence encodes a human glutathione peroxidase.

6. Adenovirus according to claim 1, characterized in that the DNA sequence is an antisense sequence whose expression makes it possible to control the expression of the gene encoding glutathione peroxidase.

7. Adenovirus according to claim 6, characterized in that it is a gene encoding an antisense RNA capable of controlling the translation of the mRNA for a glutathione peroxidase.

8. Adenovirus according to one of claims 1 to 7, characterized in that the DNA sequence is placed

under the control of signals allowing its expression in the target cells.

9. Adenovirus according to claim 8, characterized in that the expression signals are chosen from viral promoters, preferably from the E1A, MLP, CMV and RSV-LTR promoters.

10. Adenovirus according to claim 1, comprising a gDNA or cDNA sequence encoding a bovine glutathione peroxidase under the control of an RSV-LTR promoter.

11. Adenovirus according to claim 1, comprising a gDNA or cDNA sequence encoding a human glutathione peroxidase under the control of an RSV-LTR promoter.

12. Adenovirus according to one of claims 1 to 11, characterized in that it lacks the regions of its genome which are necessary for its replication in the target cell.

13. Adenovirus according to claim 12, characterized in that it comprises ITRs and a sequence allowing encapsidation, and in which the E1 gene and at least one of the E2, E4, L1-L5 genes are not functional.

14. Adenovirus according to claim 12 or 13, characterized in that it is an Ad 2 or Ad 5 type human adenovirus or a CAV-2 type canine adenovirus.

15. Use of an adenovirus according to one of claims 1 to 14, for the preparation of a pharmaceutical

composition intended for the treatment and/or prevention of neurodegenerative diseases.

16. Use according to claim 15, for the preparation of a pharmaceutical composition intended for the treatment and/or prevention of Parkinson's disease, Alzheimer's disease, Huntington's disease, ALS, trisomy 21, atherosclerosis, cardiovascular diseases, cirrhosis of the liver, diabetes, the formation of cataracts, cerebral ischaemia, cranial traumas, respiratory distress syndrome (ARDS), cancers as well as the aging process.

17. Pharmaceutical composition comprising one or more defective recombinant adenoviruses according to one of claims 1 to 15.

18. Pharmaceutical composition according to claim 17, characterized in that it is in injectable form.

19. Pharmaceutical composition according to one of claims 17 to 18, characterized in that it comprises between 10^4 and 10^{14} pfu/ml, preferably 10^6 to 10^{10} pfu/ml of defective recombinant adenoviruses.

20. Mammalian cell infected with one or more defective recombinant adenoviruses according to one of claims 1 to 14.

21. Cell according to claim 20, characterized in that it is a human cell.

22. Cell according to claim 21, characterized in that it is a human cell of the

retinal, fibroblast, myoblast, hepatocyte, endothelial cell, glial cell or keratinocyte type.

23. Implant comprising infected cells according to claims 20 to 22 and an extracellular matrix.

24. Implant according to claim 23, characterized in that the extracellular matrix comprises a gelling compound chosen preferably from collagen, gelatin, glucosaminoglycans, fibronectin, agarose and lectins.

25. Implant according to claims 23 and 24, characterized in that the extracellular matrix also comprises a support allowing anchorage of the infected cells.

26. Implant according to claim 25, characterized in that the support consists preferably of polytetrafluoroethylene fibres.

Abstract

The present invention relates to a defective adenovirus comprising at least a DNA sequence coding for all or an active part of glutathione peroxidase or a derivative thereof. It also relates to their utilisation in therapy and to the corresponding pharmaceutical compositions.

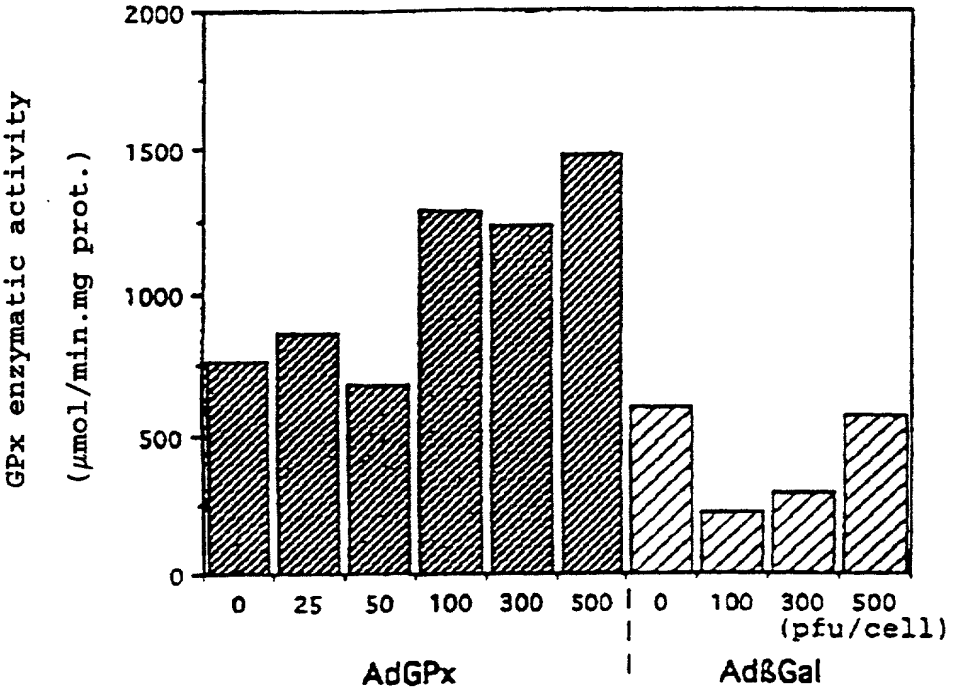
GPx enzymatic activity
(µmol/min.mg prot.)
2000
1500
1000
500
0
0
25
50
100
300
500
0
100
300
500
(pfu/cell)
AdGPx
AdßGal

Figure 1

DECLARATION AND POWER OF ATTORNEY
FOR PATENT APPLICATION

As a below named inventor, I hereby declare that my residence, post office address and citizenship are as stated below next to my name; I believe that I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought, by entry into the U.S. national stage of examination, on the invention entitled

ADENOVIRUS COMPRISING A GENE CODING FOR GLUTATHIONE PEROXIDASE

the international specification of which was filed on July 26, 1995 as Application Serial No. PCT/FR95/01002 which notice of transmission was given on February 22, 1996, by the International Bureau. I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment(s) referred to above. I acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, Section 1.56(a). I hereby claim foreign priority benefits under Title 35, United States Code, Section 119 of a foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed.

Prior Foreign Applications(s)			Priority Claimed	
FR94/09982	France	12 August 1994	X	
(Number)	(Country)	(Day/Month/Year Filed)	Yes	No
(Number)	(Country)	(Day/Month/Year Filed)	Yes	No

I hereby claim the benefit under Title 35, United States Code, Section 120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, Section 112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, Section 1.56(a) which occurred between the filing date of the prior application and the national or PCT International filing date of this application:

(Application Serial No.)	(Filing Date)	(Status-Patented, Pending or Abandoned)
(Application Serial No.)	(Filing Date)	(Status-Patented, Pending or Abandoned)

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

POWER OF ATTORNEY: I (We) hereby appoint as my (our) attorneys, with full powers of substitution and revocation, to prosecute this application and transact all business in the Patent and Trademark Office connected therewith:

Martin F. Savitzky	Reg. No. 29,699
Paul R. Darkes	Reg. No. 33,862
Raymond S. Parker, III	Reg. No. 34,893
Ross J. Oehler	Reg. No. 33,270

Send Correspondence to:
Martin F. Savitzky
Rhône-Poulenc Rorer Inc.
Legal-Patents, 3C43
P.O. Box 5093
Collegeville, PA 19426-0997

Direct Telephone Calls to: (610) 454-3816

Martine BARKATS	French
First or Sole Inventor	Citizenship
7, Rue Nicolas-Mouël	Same
Residence Address	Post Office Address
F-75005 Paris	Same
City	City
FRANCE FRX	Same
State (Zip) or Country	State (Zip) or Country
24 march 1997	Martine Barkats
Date	Signature

2-00

Jacques MALLET	French
Second Inventor	Citizenship
18, rue Charcot	Same
Residence Address	Post Office Address
F-75013 Paris	Same
City	City
FRANCE FRX	Same
State (Zip) or Country	State (Zip) or Country
Date 24 march 1997	Signature Jacques Mallet

3-00

Frédéric REVAH	French
Third Inventor	Citizenship
Bâtiment Flandre 2, 49, rue de Câtenay	Same
Residence Address	Post Office Address
F-92160 Antony	Same
City	City
FRANCE FRX	Same
State (Zip) or Country	State (Zip) or Country
Date 4 April 1997	Signature Frederic Revah